REDUCING PROPERTIES OF NORMAL AND ABNORMAL MILK AND THEIR IMPORTANCE IN BACTERIOLOGICAL GRADING OF MILK

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CONTENTS

I. INTRODUCTION

Milk constitutes a complicated biological system embracing many redox systems of different concentrations and compositions. In addition to the natural redox systems of the milk, infected milk contains the redox systems of the microorganisms concerned, with properties varying according to the composition of the flora.

Reviews have already been written on specialized aspects concerning the different dye tests in connection with the bacteriological grading of milk (19). This review, however, is concerned mainly with the biological systems constituted by the milk and affecting the redox processes by catalysis, inhibition, poising, etc. A number of subsidiary problems closely related to the main issue will also be reviewed in order to complete the picture.

II. REDUCING PROPERTIES OF NORMAL AND MASTITIS MILK

Much discussion has taken place on the types of reaction that will bring about the dye reduction in milk (1, 2, 18). The observations reported in the literature are nearly all derived from investigations on milk in which active bacterial multiplication has taken place. Some observations suggest, however, that there are reasons for believing that the reduction of methylene blue and especially resazurin can be affected by abnormal states of the udder (5). In the work that is to be summarized here, most attention has been devoted to ascertain the *types* of reducing systems active in milk, and to establish the *differences* in reducing properties between normal and mastitis milk. Most of the studies have been made on aseptically drawn milk, but some subsidiary problems that have been examined also concern ordinary producer milk.

A. Redox Systems of Normal and Mastitis Milk

In normal milk at least four reducing systems are known: (a) A system which under strictly anaerobic conditions is capable of reducing methylene blue in raw milk. (b) One system, also present in raw milk, which is capable of reducing methylene blue aerobically in the presence of formaldehyde. (c) One reducing system present in autoclaved milk which is revealed under anaerobic conditions. (d) One reducing system formed both in raw and boiled milk, which is active under anaerobic conditions in the presence of light.

These systems have all been studied by the methylene blue reduction test. All redox indicators such as methylene blue, resazurin, etc., have a range of about 100 mv, which covers the change from colored to colorless form. In my investigations I have followed the changes in redox potential by direct potentiometry, the methylene blue and resazurin tests being carried out in addition for purposes of comparison (10). By this potentiometric method it was possible to demonstrate that aseptically drawn milk from udder quarters

in which the diagnosis of mastitis had been established did contain a reducing system not comparable with those that are to be found in normal milk. This system was strong enough to produce a fall in potential varying between 50 to 400 mv, when tested aerobically at 37 C. Under the same conditions normal milk produced no fall in potential (11).

This reducing system of mastitis milk or parts of it was found to be associated with the fat, and was destroyed by heating to 85 C for 5 min.

B. Role of Enzymes, Hydrogen Donators, and Precursors in Redox Properties of Normal and Mastitis Milk

To obtain some idea of what redox systems may be active in milk, and thus to distinguish between normal and mastitis milk, the following must be borne in mind.

First, the redox systems in the two types of milk may be different. Thus, that of normal aseptically drawn milk seems to be in a state of equilibrium at 37 C, since the potential is then constant provided that no additional reducing or oxidizing systems bring about a change (for example, variation in oxygen content or growth of bacteria), whereas the redox system of mastitis milk is not in a state of equilibrium at this temperature, similar treatment resulting in a fall in potential.

Second, the redox systems of the two types of milk may, on the other hand, be similar but present in different concentrations, the system of mastitis milk being such that, under the conditions prevailing, fall in potential occurs.

One of the lines of approach to this problem was to list the various redox systems known and believed to be present in milk, and to attempt to assess whether they might be responsible for the fall in potential noted in mastitis milk. The following are the oxidation-reduction systems and substances that may occur in milk.

Any redox system is characterized by the potential found when 50 per cent of the substance is in oxidized form and 50 per cent in reduced form. This value is termed E_0. In cases of redox systems involving H^+ ions, *i.e.*, most biological systems, the expression E_0' is commonly used (6). The E_0' value for mastitis milk arrived at was 0.172 v (pH 6.8 to 7.0).

With the exception of cytochrome *c* (which has $E_0' = 0.27$ v) all the systems listed in table 1 had an E_0 lower than that obtained for mastitis milk, and might therefore contribute to the fall in potential in mastitis milk.

Another procedure for establishing what redox systems are active would be the following. Both normal and mastitis milk contain dehydrogenases which, alone or with others, are capable of causing reduction in the presence of suitable hydrogen donors and acceptors.

Different hydrogen donors were added to normal and mastitis milk, and the change in redox potential was traced. Certain of the added substances were capable of acting as donors in the reducing enzyme systems of the milk: all of these acting directly as donors for the enzyme xanthine oxidase[1] were found to cause marked fall in potential; and adenine and guanine also produced fall in potential in mastitis milk but only adenine did so in normal milk. This shows that both types of milk contain adenase, and that mastitis milk contains also guanase (13). Adenase and guanase are not actually oxidation-reduction enzymes, but they produce donors for the redox enzyme, xanthine oxidase. This effect of the enzymes adenase and guanase, of producing donors from precursor substances for the redox system containing xanthine oxidase, was later established as a very important factor in bringing about the reducing capacity of mastitis milk. The experiments performed to characterize the redox systems of normal and abnormal milk showed that the only ones that cause a fall in potential are those that act directly as donors for xanthine oxidase or indirectly after being converted to donors for xanthine oxidase.

Furthermore, it was found that both normal and mastitis milk react in the same manner, with regard to all substances examined except for guanine. In other comprehensive experiments carried out to characterize quantitatively the reducing properties of aseptically drawn normal and mastitis milk the following was found. The fundamental difference between the two types of milk seems to be that in mastitis milk the donors and/or the donor precursors for the xanthine oxidase system are present in a concentration that results in a fall in potential at 37 C, whereas the

[1] It was stated in earlier experiments that mastitis milk reduced the Schardinger solution, which contains the donor for xanthine oxidase, formaldehyde, and methylene blue, much faster than normal milk.

TABLE 1

Oxidation-reduction systems and substances possibly occurring in milk

Enzyme	Substrate	E_0' at pH 7
		v
A. Dehydrogenases		
Alcohol	Ethyl alcohol	−0.090–0.16
Lactic	Lactic acid	−0.18
Malic	Malic acid	−0.10
Glucose	Glucose	−0.45
Glutamic	Glutamic acid	−0.03
Succinic	Succinic acid	0.01
Aldehyde	Hypoxanthine, certain aldehydes	−0.37
Xanthine oxidase	Xanthine	−0.37
B. Oxidases		
Cytochrome	Reduced cytochrome *c*	0.27
Ascorbic	Ascorbic acid	0.078–0.05
C. Peroxidases (require H_2O_2)		
Milk	Aromatic amines and special phenols	
Cytochrome *c*	Reduced cytochrome *c*	0.27
Riboflavin		−0.208
Glutathione		−0.233

concentration of those in normal milk is too low for this to happen.

The determination of total content in milk of various donors and donor precursors for xanthine oxidase is very difficult. It is possible, however, to determine the nature and quantity of the products obtained after that fall in potential has taken place, *e.g.*, in mastitis milk, and to compare the results with those obtained for normal milk.

By using such methods it has been found that in mastitis milk after fall in potential the content of uric acid rises about 0.10 mg per ml. In normal milk no fall in potential and no change in uric acid content was found. The content of other acids, *e.g.*, valeric, butyric, propionic, and acetic, was the same in the two types of milk, which shows that the donors in mastitis milk cannot have consisted of aldehydes.

It was also possible to demonstrate the existence of a higher concentration of the enzyme xanthine oxidase in mastitis milk than in normal milk. This was evident from the facts that (a) in the presence of a surplus of formaldehyde the K_m values were higher for mastitis milk than for normal milk, and (b) the Schardinger solution was reduced more quickly by mastitis milk than by normal milk (12).

Thus it was shown that the difference in reducing properties of the two types of milk mainly depends upon the following factors. Mastitis milk obtained under aseptic conditions and with a low total bacterial content possesses a reducing property not shown by normal milk, which can be demonstrated by potentiometric determination at 37 C. This reducing property is due to the presence of substrates (chiefly in the form of precursors that, via appropriate enzymes, produce hydrogen donors in the form of hypoxanthine and xanthine) for xanthine oxidase in a concentration high enough to render possible a fall in potential. Normal milk also contains enzymes that allow a fall in potential on condition that donators or donator precursors (not guanine) are added in sufficient amounts.

C. Influence of Methylene Blue, Fat Content, and Leukocytes on Development of Reducing Capacity of Milk

In studying oxidation-reduction processes in milk, due regard must be paid to the many factors that may, in one way or other, influence the reduction changes taking place. Among such factors, those above-mentioned are of special interest. Neither methylene blue nor other redox indicators can be regarded as inert indicators, as they are redox systems themselves; the fat content is known to affect the methylene blue reduction of normal milk; and finally it has often been suggested that the reducing properties of mastitis milk of low bacterial content are due to the high leukocyte content of this milk. In the work under review, addition of methylene blue to mastitis milk was found to hasten the fall in potential of this milk. When donors for xanthine oxidase were added to normal milk so that fall in potential was made possible, this fall was also hastened by the addition of methylene blue. Several different explanations of this phenomenon were set out. The following, however, seems to be the most likely. The enzyme, xanthine oxidase,

contains the prosthetic group adenine-flavine-dinucleotide. Flavoproteins are autoxidizable by molecular oxygen. The rate of oxidation is known to be very slow, however, but it may be greatly increased by adding a reversible dye, such as methylene blue, which serves as a carrier between the flavoprotein and oxygen.

It was also found that a high fat content was associated with a more rapid fall of the potential in the mastitis milk. This is evidently due to the facts (a) that the xanthine oxidase is bound to the fat and is therefore present in greater concentration in samples with high fat content, and (b) that the surface upon which the reactions can occur is greater in rich than in poor milk. Bearing in mind the significance of the amount of donors and donor precursors plus their enzymes in the development of a fall in potential, the findings indicate that the concentration of these substances also increases with increasing fat content. It is an intriguing possibility that the entire xanthine-oxidase system may be mainly concentrated to the surface membrane of the fat globules.

It has often been suggested that the enhanced reducing capacity of mastitis milk of low bacterial content is due to the high leukocyte content in this milk. It is shown, however, that the leukocytes cannot be directly responsible for the phenomenon. The leukocytes themselves were found to have no reducing capacity, as was shown by adding washed leukocytes from one infected and one normal quarter of an udder (obtained by centrifuging 50 ml of milk) to 20 ml of pasteurized normal milk. On testing, neither sample showed any fall in potential. An indirect relationship cannot be excluded, however, because the conditions resulting in collection of leukocytes in the udder, and secondarily also in the milk, may very well also bring about the transference of increased quantities of other substances from blood to milk. The leukocytes, too, might contribute by enzymatic disintegration to an increase in content of precursors in the form of nucleic acid, or they might influence the reduction indirectly by raising the total enzymatic activity in the milk. A phenomenon of this nature may well commence even before the milk has left the udder, especially since addition of leukocytes *in vitro* failed to produce such an effect in normal milk.

III. REDUCING PROPERTIES OF MASTITIS ORGANISMS

Among the organisms giving rise to mastitis the commonest are streptococci, staphylococci, and coliform microorganisms. The streptococci are held responsible for at least 85 per cent of cases of chronic mastitis.

In contradistinction to the lactic-acid streptococci, the mastitis streptococci are as a rule hemolytic. Hewitt (6) has shown that a rapid fall in potential may occur in a bacterial culture of hemolytic streptococci if catalase is added. Since mastitis milk contains relatively large quantities of catalase, it is quite conceivable that it constitutes a good medium for the development of hemolytic microorganisms. According to Hammer (4), Hobbs (7), and others, the hemolytic microorganisms causing mastitis have extremely weak reducing properties, however. It is also well known that freshly drawn milk has bactericidal properties (4).

In the investigation described, and in the methylene blue test applied in practice, the milk is examined at a temperature of 37 C. At this temperature the pathogenic bacteria may conceivably overcome the bactericidal properties of the milk and assert themselves. The number of hemolytic organisms in aseptically drawn milk from single quarters was counted on blood agar. In all, 201 samples from single quarters were investigated. These included 11 samples from animals with confirmed mastitis.

The number of hemolytic microorganisms proved to be very small in the mastitis milk samples. Only in one case were there more than 1000 per ml. It was, however, possible to demonstrate the occurrence of a few hemolytic microorganisms in 91 of the remaining 190 samples of normal milk.

How marked a fall in potential, then, can the mastitis bacteria cause in milk at 37 C? To answer this question, the fall in oxidation-reduction potential produced by pure cultures of mastitis-provoking microorganisms inoculated into aseptically drawn normal milk was determined. Thus the reducing capacity in milk of *Escherichia coli* (2 strains), *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Corynebacterium pyogenes*, *Staphylococcus aureus* (2 strains), *Streptococcus agalactiae* γ, *Streptococcus dysgalactiae*, and *Streptococcus uberis* was determined. It was found that about 5000 organisms per ml were necessary to obtain a fall in potential (14).

The mastitis streptococci studied (with the exception of *S. uberis*, which produced some fall in potential) produced no fall in potential, even when inoculated in such large quantities, and the same was true of *C. pyogenes*. The two strains of

staphylococci studied had relatively weak reducing capacity. Finally, the coliform organisms showed marked reducing capacity. Thus in cases of mastitis due to *E. coli* and possibly also to staphylococci, it must be borne in mind that the reducing capacity of the mastitis milk at 37 C may be partly of bacterial origin if the pathogenic organisms are present in great numbers.

IV. MEAN OXIDATION-REDUCTION POTENTIAL OF NORMAL AND ABNORMAL ASEPTICALLY DRAWN MILK

In the investigations on the reducing properties of normal and mastitis milk, the samples were taken from stock which was under the constant control of a veterinarian. During the period 1953 to 1955, aseptically drawn samples were taken from different quarters of the udder. The E_h, pH, reductase test, Schardinger test, fat content, catalase number, and leukocyte count were determined at the laboratory. This material was classified according to cell content and catalase index, and arranged in 5 groups (table 2)(15).

It was thus established that no marked change in E_h takes place in samples with an increased catalase index or raised cell count. A somewhat lower E_h is found in samples with both high catalase index and high leukocyte content. The results also show that normal, aseptically drawn milk from the investigated herd had a mean E_h value of 324 ± 2 mv. Milk showing a fall in potential after 6 hr (group V) had a distinctly lower E_h value at the first determination. Further, it was found that (a) during the period immediately after calving, the E_h value of the milk was relatively high, and then sank to about 310 mv, at which level it seemed to remain relatively constant for the remainder of the lactation period; (b) the E_h value of the milk varied with the seasons of the year, being minimum in the winter and maximum during the latter part of the summer; (c) it was not possible to observe any variations in E_h between the different udder quarters or (with one exception) between twin cows.

TABLE 2

Initial E_h values of milk of varying cell count and catalase index

Group	Catalase Index in Milk	Leukocyte Count in Milk	No. of Samples	E_h (First Reading)
	units/ml	*cells/ml*		*mv*
I	<5	<500,000	441	324 ± 2
II	<5	>500,000	22	333 ± 6
III	>5	<500,000	16	337 ± 13
IV	>5	>500,000	19	319 ± 7
V	>5	>500,000*	31	285 ± 10

* Mastitis milk, with fall in potential after 6 hr.

V. SOME CHARACTERISTICS OF FARM MILK

In order to obtain as complete a picture as possible of the different factors contributing to the reducing capacity of milk, studies on the reducing properties of the bacteria occurring in milk were also carried out. Our knowledge on this subject is based mainly on data concerning the aggregate reducing capacity of the different species of bacteria present in a certain milk plus the reducing properties of the milk itself.

Hammer (4) states that *Streptococcus lactis* reduces methylene blue rapidly, and Bendixen and Ellington (3) have found proteolytic, alkali-forming, inert, lactic-acid-producing organisms to be as effective in reducing methylene blue as are ordinary lactic-acid bacteria. At 37 C it is, as Wilson (19) asserts, doubtful that the lactic streptococci are as important as the coliform bacteria. In summary it would seem to be generally accepted that *S. lactis* is the organism to which most of the reducing capacity in normal milk is to be ascribed. In this connection and with reference to earlier investigations (11) the probable effect of admixture of mastitis milk to ordinary milk upon the fall in potential may be mentioned, namely an inhibitory effect upon the development of lactic-acid bacteria. The result of, *e.g.*, the reductase test in a milk with admixture of mastitis milk is therefore very complicated. One property of the mastitis milk, *viz.*, its own reducing capacity, tends to shorten the reduction time for the reductase test, whereas another property, its inhibitory action upon the lactic-acid bacteria, has the opposite effect.

The aim was to contribute to a characterization of the microflora in farm milk in respect of the reducing capacities of the commonest species of this flora, and to ascertain the connections existing between the reducing capacity of the single bacterial species and that of the milk itself. The following analyses were carried out on samples of ordinary farm milk. Variations in E_h during 6 hr at 37 C, reductase test, resazurin test, Schardinger test, catalase number, leukocyte count, fat content, and pH value. The samples were plated out on tryptone-glucose agar, violet-red-bile agar,

and blood agar. The total colonies were counted after incubation. In all, 93 different samples were examined, and the following results obtained.

A relationship was found to exist between the reduction time in the reductase test and the number of coliform bacteria occurring in the same sample, and also between the reduction of Schardinger solution (xanthine oxidase content) and resazurin solution on the one hand and high leukocyte count and catalase index on the other. A clear correlation was found between the total bacterial content of the samples and the potential-lowering capacity (16). The connection between the total number of bacteria in the milk and the percentage of bacteria possessing marked reducing capacity, as shown when the calculations were applied to different organisms isolated from the total flora, was not pronounced. Thus, to judge from these results, the increase of the total flora in a sample of farm milk appears not to be specifically correlated to an increase of the fraction having the strongest reducing capacity.

VI. POSSIBILITIES OF ANALYZING BULK MILK FOR CONTAMINATION WITH MASTITIS MILK

The literature contains many reports of methods for veterinary analysis of milk from cows suffering from mastitis (5, 8, 9). Practically nothing has been written concerning the problem of determining whether bulk milk at the receiving station is from healthy cows or is mixed with mastitis milk, however.

It has been stated that mastitis milk, because of its enzyme activity, produced an abacterial reduction. It therefore seemed interesting to attempt to distinguish normal and abnormal milk, starting from the differences in reduction due to the xanthine oxidase system (17).

The xanthine oxidase activity parallels the fat content, *i.e.*, a shorter reduction time is found in milk with a high fat content. The reduction times in the Schardinger test in aseptically drawn normal milk with varying fat content and in mastitis milk is shown in table 3.

The differences in the capacity to reduce Schardinger solution demonstrated in table 3 are significant for series V and VI.

The possibility of devising a simple test for differentiating farm milk into normal and mastitis milk on the basis of the observed differences in reduction time with Schardinger solution will depend on the following factors: (a) whether the bacterial metabolism and the consequent reduction in the milk can be eliminated and (b) whether the amount of mastitis milk detectable is of an order of magnitude that makes the sample valuable from a practical point of view.

TABLE 3

Reduction times in Schardinger test in normal milk with varying fat content and in mastitis milk

Series	Fat Content	No. of Samples	Mean Reduction Time for Schardinger Solution	Standard Error
	%		*min*	*min*
	Normal milk:			
I	0.0–1.0	79	182.8	5.23
II	1.1–2.0	181	101.9	3.00
III	2.1–3.0	131	66.0	2.12
IV	3.1–4.0	69	47.1	1.53
V	>4.0	21	38.8	3.84
VI	Mastitis milk with varying fat content	31	23.8	2.28

After comprehensive studies it was possible to make the following conclusions regarding these two conditions.

1. The antiseptic, toluene, did not to any significant degree influence the reduction time with Schardinger solution. This substance checked the bacterial development in the milk very effectively. Thymol did not check the bacterial activity. The third substance studied, chloroform, completely inhibited fall in potential in mastitis milk due to enzymatic action.

2. The amount of mastitis milk that can be detected in a mixed sample will of course depend upon the reduction capacity of the mastitis milk itself, and upon the fat content of the normal milk. In the experiments performed it was possible to show a distinct fall in potential in connection with the admixture of 10 per cent mastitis milk, however.

The problem with bulk milk is that it often has a high fat content. The differences in reduction time between normal and mastitis milk when the Schardinger test plus toluene is used will therefore not be so pronounced.

The experiments so far carried out are only of a preliminary nature, but the Schardinger test seems promising, especially in milk of low fat content, for determining whether the milk derives from healthy or infected stock.

Finally, the difference in the incidence of guanase might form the basis of a test for detecting contamination. Further experiments have shown that the resazurin test, applied after inhibiting bacterial activity by addition of toluene, holds great promise as a simple method of detecting contamination of bulk milk with mastitis milk.

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